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APPLICANT: MARSHALL, William E.
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TITLE: METHODS AND COMPOSITIONS FOR MODULATING
IMMUNE SYSTEMS OF ANIMALS

GRP./A.U.: 1645
EXAMINER: ZEMAN, R.
CONF. NO.: 1897
DOCKET NO: P01936US5

**132 DECLARATION OF
WILLIAM E. MARSHALL**

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, William E. Marshall hereby declare the following.

1. I am the inventor on the above-identified case and am familiar with the prosecution including the Office Action dated May 30, 2003.
2. My background includes a Ph.D. in biochemistry from the University of Illinois, post-doctoral training at Uppsala University and Cambridge University, assistant professor of biochemistry at the University of Minnesota, director of technology development at General Foods Corp., president of the Microbial Genetics Division of Pioneer Hi-Bred International, member of the Iowa Academy of Sciences, chairman of the National Agricultural Research and Extension Users Advisory Board of the U.S. Congress, member of the advisory panel on biotechnology to the Office of Technology Assessment of the U.S. Congress, member of the advisory panel on intellectual property to the GATT, and associate professor of microbiology and immunology at the New York Medical College.

3. I understand that the Examiner has placed rejections on my application based upon one or more of the following references: De Vuyst et al., 1996 142 p. 817-827; Nanji, U.S. Patent No. 5,413,785; and Perdigon et al., 1990 J Food Production 53 (5) p. 404-410. This application details scientific argument and experimental evidence to refute the Examiner's contentions. The Examiner states that "De Vuyst et al. disclose methods of producing low molecular weight proteins from bacteria by subjecting them to a number of stresses. By definition, these proteins are stress response factors. De Vuyst et al. further disclose that these bacteriocins are able to kill or harm other bacterial species and suggest the use of said bacteriocins as food additives. Consequently it would have been obvious to one of ordinary skill in the art at the time the invention was made to have followed the suggestion of De Vuyst et al." Further, the Examiner states "Nanji discloses the administration of lactic acid bacteria to humans, livestock and other animals for protection against endotoxin-mediated shock. Therefore, it would have been obvious to one of ordinary skill in the art to use the bacteriocins by De Vuyst et al. in the treatment of methodologies of Nanji in order to take advantage of the immune enhancing effects of the bacteriocins while minimizing the complications associated with introducing a bacterial strain into the normal flora of an animal." Finally, the Examiner states "the teachings of De Vuyst et al. in view of Perdigon et al. disclose the use of lactic acid bacteria and the proteins produced therein as immunogens and adjuvants in the generation of protection from enteropathogens. It would have been obvious to one of ordinary skill in the art at the time the invention was made to use low molecular weight proteins disclosed by De Vuyst et al. as adjuvants for the induction of a immune response to another co-administered pathogen since Perdigon et al discusses the use of lactic acid bacteria (and the proteins produced by said bacteria) as adjuvants for enteropathogens (an increased immune response to said enteropathogens was also disclosed) and De Vuyst et al. disclose that proteins produced by lactic acid bacteria have an immunomodulatory effect." The present application is distinguishable from the cited references because De Vuyst et al, Nanji, and Perdigon et al. claim proteins. In contrast, I have determined that the SRFs are

oligoribonucleotides. Described herein are the methods and results used to identify the nature of the present invention's intracellular solutes.

4. Attached is Figure 4 from U.S. Patent No. 5,840,318 representing the separation of the intracellular solutes. There is no evidence of the presence of proteins or peptides among the claimed invention's intracellular solutes therefore, I assert the present invention's intracellular solutes are not bacteriocins (proteins/peptides) as suggested by the Examiner.

Figure 4 is a drawing of a Sephadex G-10 separation of the intracellular solutes (SRFs) typically released by the stressing of bacteria. It is the same Figure 4 in U.S. Patent No. 5,840,318, Methods and Compositions for Modulating Immune Systems of Animals, Marshall and Hoffmann. It shows that the <10kDa SRFs are a mixture of small components having maximum absorbance at 254nm, not 280 nm indicating that they are nucleotides, not proteins or peptides.

5. Also attached herewith and for the Examiner's consideration is a Xerox copy of a page from my lab notebook obtained from experiments conducted to refute several of the Examiner's contentions. The copy displays a figure (Figure 5) representing the separation of the intracellular solutes on HPLC (high pressure liquid chromatography) which indicate 13 peaks or components.

Figure 5 is a Xerox copy of a machine tracing showing the separation of the intracellular solutes (SRFs) on HPLC (high pressure liquid chromatography). The X-axis is the time of elution of the components in minutes; the Y-axis is the absorbance at 220nm. The effluents corresponding to the peaks was further examined by UV absorbance and showed maxima at 254 nm, typical of nucleotides and not 280 nm as one would expect from proteins. There is no evidence of the presence of proteins or peptides among the claimed invention's intracellular solutes therefore, I assert the present invention's intracellular solutes are not bacteriocins (proteins/peptides) as suggested by the Examiner. The tracing indicates 13 peaks or components in two groupings. Since the first group of nucleotides appearing as Peaks 3-6 were eluted immediately with an aqueous solvent, they are hydrophilic. The second group of peaks, labeled 19-23 are hydrophobic since they

were eluted with a solvent that was 50% methyl cyanide and 0.1% trifluoro acetic acid. The claimed invention's intracellular solutes are a mixture of 10-13 major nucleotides, smaller than 10 kDa. Approximately half are hydrophobic and half are hydrophilic.

6. Attached herewith and for the Examiner's consideration is a Xerox copy of a page from my lab notebook obtained from experiments conducted to refute several of the Examiner's contentions.

Figure 6 is a photo and hand drawn table of a Thin Layer Chromatogram (TLC) of the SRFs less than 10 kDa released from *L. monocytogenes* and detected by UV light at 254nm. The large dark spot at the origin in Lane 4 represents Peak I from Sephadex. Staying at the origin of application indicates that is insoluble in the aqueous alcohol solvent and probably large, i.e., it corresponds to the hydrophobic group seen on the HPLC. The spots labeled A-D are components of Sephadex Peaks II-IV and correspond to smaller, hydrophilic nucleotides and the free base, uracil. The presence of uracil and the absence of thymine (TLC not shown) indicates that the nucleotides are from RNA, not DNA.

7. The Examiner states "[b]y definition, these proteins are stress response factors." Attached herewith and for the Examiner's consideration is a Xerox copy of a page from my lab notebook that presents a figure (Figure 7) that demonstrates my assertion that the present invention's SRFs are ribonucleotides, not proteins or peptides, smaller than 10kDa. This figure also represents my assertion that there is no evidence of the presence of proteins or peptides among the claimed invention's intracellular solutes as suggested by the Examiner.

Figure 7 is a photo and drawing demonstrating that SRFs less than 10 kDa from *L. caseii* are similar to those released by *L. monocytogenes*. It indicates that some oligoribonucleotides are hydrophobic and large while others are hydrophilic and small. It also shows that oligoribonucleotides are repeatedly released during exposures to pH neutral buffers. In Lanes 12 and 13 are the oligoribonucleotides from two washings of *L. caseii*.

8. Our invention is not limited to only stressing lactic acid bacteria. We have demonstrated that the phenomenon of stressed bacteria releasing immune-activating SRFs less than 10 kDa is also observed in Gram-negative bacteria, *S. typhimurium*, *E. coli*, and *K. pneumoniae*, a strict anaerobe, *B. coryneforme*, and the gram-positive pathogens, *S. aureus*, *L. monocytogenes*, and *S. pyogenes*. The use of harmless generally regarded as safe lactic acid bacteria has been emphasized only for purposes of minimizing manufacturing costs and allowing the technology to be practiced as a cottage industry. Further, the present invention is specifically related to how the bacteria use the aforementioned intracellular solutes to create a dormant state and how the immune system has adapted an alert response to their sudden appearance. The claimed invention and the §132 Declarations submitted herewith and on March 17, 2003 show that the SRFs of the present invention are not proteins, polypeptides, or peptides and they do not contain amino acids but rather are oligoribonucleotides. Further, the SRFs of the invention do not alter growth patterns or the growth rates of other bacteria. Therefore, it is respectfully submitted the cited references do not teach or suggest the unique method of the claimed invention.

9. SRFs are a mixture of compounds having an absorption maximum at 254 nm, indicating the presence of nucleotides, not proteins. Proteins have a maximum at 280 nm.

10. The prior art of De Vuyst et al., Nanji and Perdigon as summarized by the Examiner teaches that proteins (bacteriocins) released by bacteria can kill other bacteria and, can also act as adjuvants. In contrast, the SRFs in my invention are not proteins, polypeptides or peptides; they do not contain amino acids but rather they are oligoribonucleotides. Further, the SRFs do not alter the growth patterns or the growth rates of other bacteria. I also assert that the earlier § 132 Declaration of March 17, 2003 showed that the released intracellular solutes of the claimed invention lacked any bacteriocin-like activity.

11. In addition, re-submitted herewith and for the Examiner's consideration are 3 photographs obtained from experiments conducted to refute several of the Examiner's

contentions. These photographs were submitted in the previous 132 Declaration of March 17, 2003 and noted by the Examiner as unreadable.

The protocols are detailed beneath the photos. The results show that neither total SRFs nor SRFs <10 kDa prepared from stressing 5 strains of *Lactobacillus plantarum*, 2 strains of *Enterococcus faecium*, *L. casei*, *L. acidophilus*, and *Listeria monocytogenes* contain bacteriocins. The release of SRFs was induced by transferring them from their growth media into Dulbecco's LPS-free phosphate-buffered-saline, pH 7.3 for 20 hours, at 37°C unless specified otherwise.

Figure 1 is a photo demonstrating that neither total SRFs nor those <10 kDa released by stressing bacteria results in the production of bacteriocins.

Figure 2 is a photo demonstrating that stressing higher levels of bacteria, or stressing bacteria twice or heat-killing bacteria does not result in the production of bacteriocins.

Figure 3 is a photo demonstrating that neither total SRFs nor those <10 kDa released by stressing bacteria results in the production of bacteriocins tested against a lawn of a mixture of lactic-acid bacteria.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date: 9-25-2003

William E. Marshall
William E. Marshall

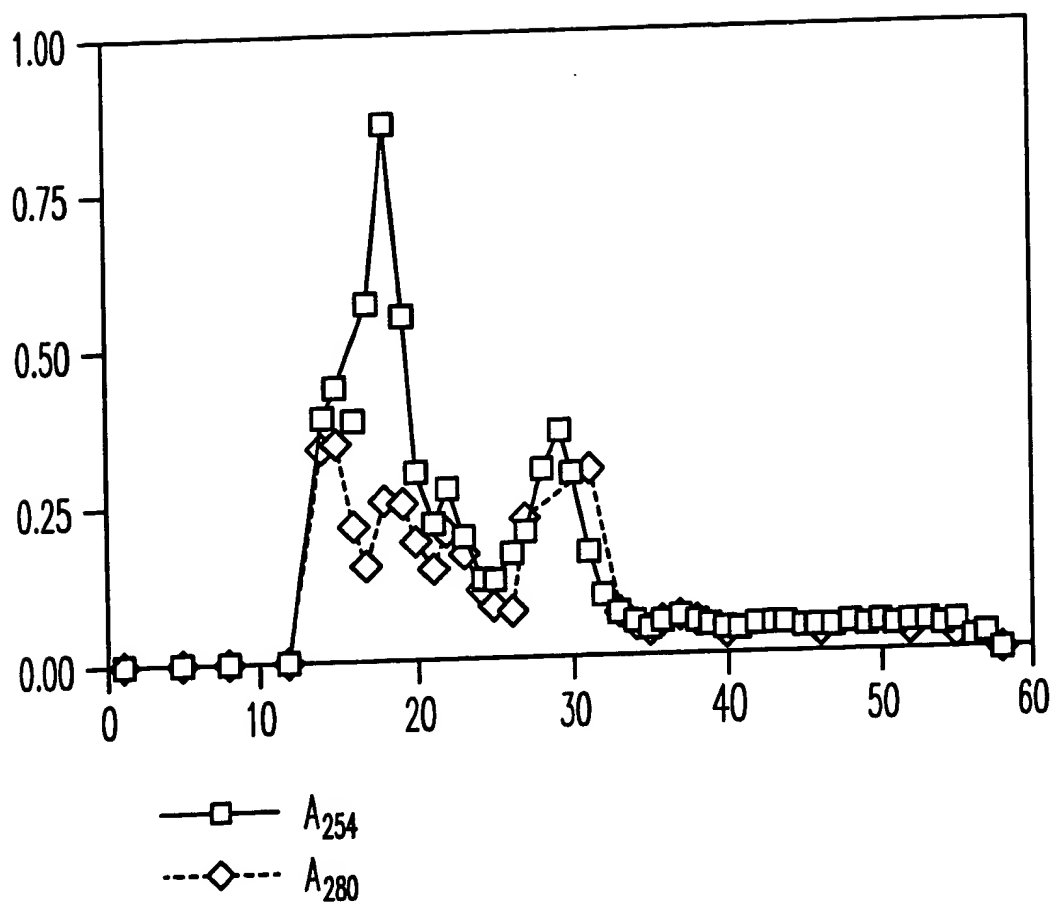


Figure 4

Shows the <10 kDa SRFs released by a direct-fed-microbial or animal probiotic commercially marketed which indicates the presence of macrophage-activating oligomers.

HPLC

6.9.96

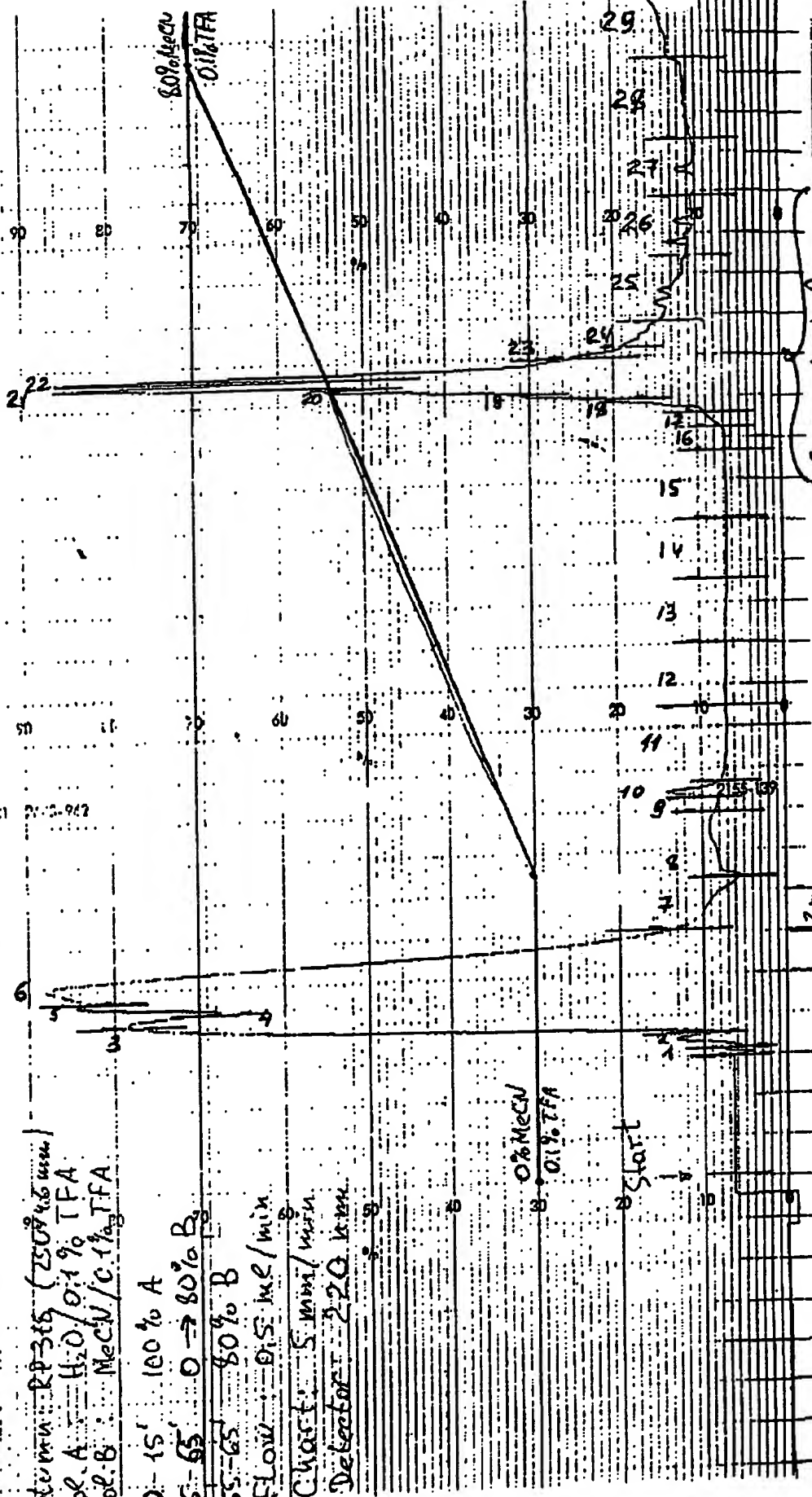
column: RP-315 (250 x 4.6 mm)
 col. A: H₂O / 0.1% TFA
 col. B: MeCN / 0.1% TFA

0-15' 100% A
 5-65' 0 → 80% B
 55-65' 80% B

Flow: 0.5 mL/min

Chart: 5 mm/min

Detector: 220 nm



Hydrophilic group

Hydrophobic group

Sample #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	25	30	50	30	34	125	150	15	3	2	15	1	1	1	1	1	1	1	1	1	1

FIGURE 5

FIGURE 6

SRFs <10 kDa from *L. monocytogenes* Are Oligoribonucleotides

PROCEDURE:

Glass plates coated with silica were purchased from E-M Science, Dusseldorf, Germany. Solvent was n-Propanol:ammonium hydroxide:water, 6:3:1, by volume according to Grippo, P., M. Iaccarino, M. Rossi, and E. Scarano. 1965. Thin-layer chromatography of nucleotides, nucleosides and nucleic acid bases. *Biochim. Biophys. Acta* 65:95: 1-7.

Aliquots of 10 μ l of the following were spotted in the silica on a line 2.5 cm from the bottom of the plate. They were allowed to dry for an hour at room temperature before the plate was put into a closed chamber containing the solvent, about 0.5 cm deep. The solvent was allowed to develop for 3½ hr up the plate for 13.5 cm at room temperature. The plate was removed from the chamber, dried at room temperature and photographed under an ultraviolet light of absorbance 254 nm.

Spotted in lanes:

1. pH neutral buffer
2. SRFs >10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
3. SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
4. Sephadex Peak I of SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
5. Sephadex Peak II SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
6. Sephadex Peak III SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
7. Sephadex Peak IV SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
8. Cyclic AMP standard
9. dibutyl cyclic AMP standard
10. AMP
11. CMP – cytidine monophosphate standard
12. GMP – guanosine monophosphate standard
13. UMP – uridine monophosphate standard
14. free uracil
15. vacant
16. combination of dibutyl cyclic AMP, Cyclic AMP and AMP
17. vacant
18. combination of AMP, GMP, CMP and uracil

RESULTS & CONCLUSIONS:

L. monocytogenes releases ribonucleotides during a 12-hr exposure to pH neutral buffer. The fraction <10kDa contains oligoribonucleotides.

Silica plate 2 NH₄OH

Results:

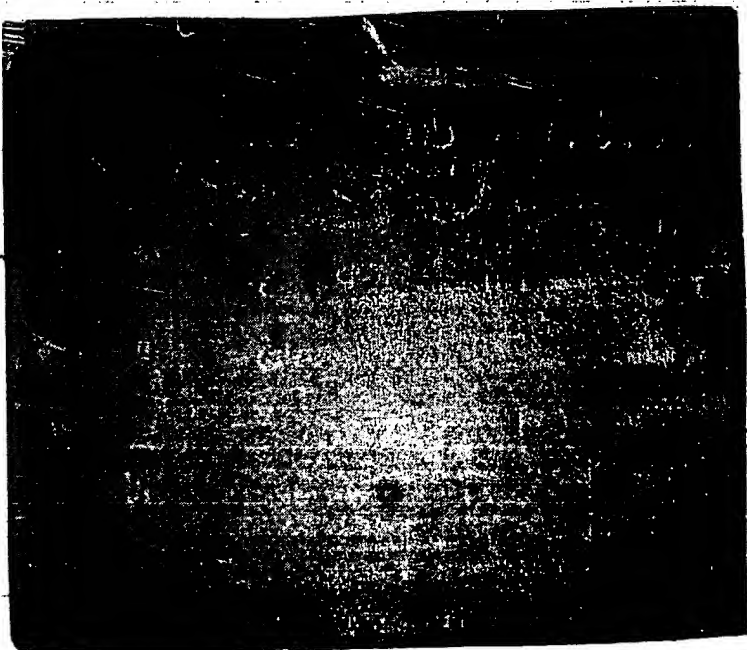
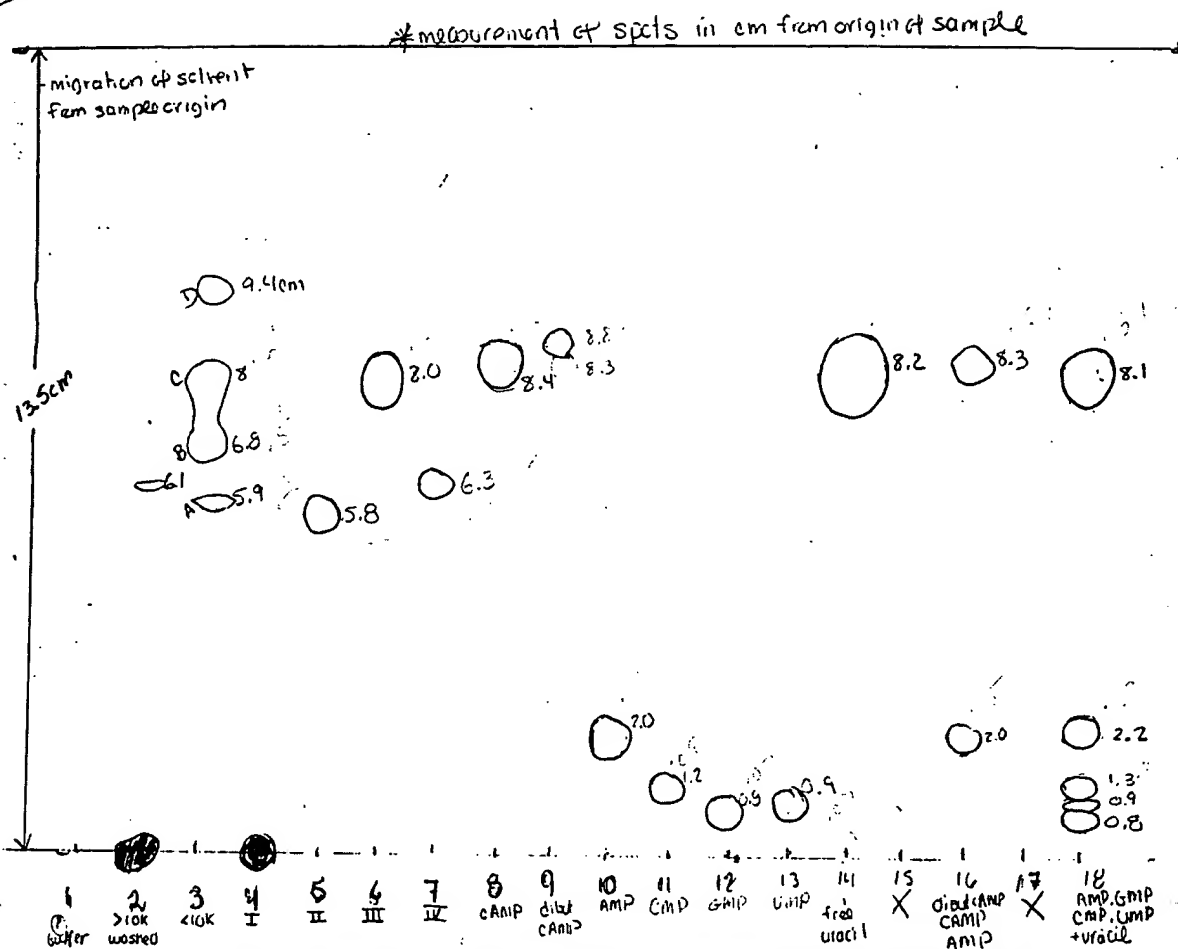


FIGURE 7
SRFs <10 kDa from *L. casei* Are Oligoribonucleotides,
Similar to those Released by *L. monocytogenes*.

PROCEDURE:

Glass plates coated with silica were purchased from Boehringer-Mannheim. Solvent was n-Propanol:ammonium hydroxide:water, 6:3:1, by volume according to Grippo, P., M. Iaccarino, M. Rossi, and E. Scarano. 1965. Thin-layer chromatography of nucleotides, nucleosides and nucleic acid bases. *Biochim. Biophys. Acta* 65:95: 1-7.

Aliquots of 10 μ l of the following were spotted in the silica on a line 2.5 cm from the bottom of the plate. They were allowed to dry for an hour at room temperature before the plate was put into a closed chamber containing the solvent, about 0.5 cm deep. The solvent was allowed to develop for 3½ hr up the plate for 13.5 cm at room temperature. The plate was removed from the chamber, dried at room temperature and photographed under an ultraviolet light of absorbance 254 nm.

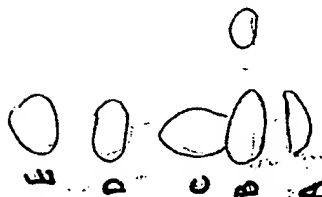
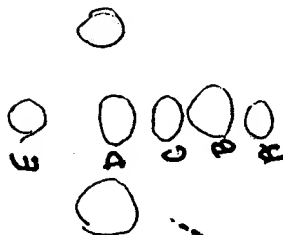
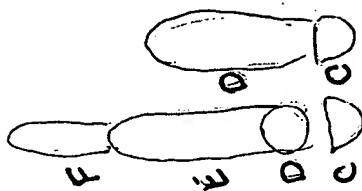
Spotted in Lanes:

1. Total SRFs released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
2. Sephadex Peak II SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
3. Sephadex Peak III SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
4. Total SRFs released by 10^9 colony-forming-units of *L. monocytogenes* during 3½ hr in pH neutral buffer after an initial 12 hr exposure in pH neutral buffer.
5. Cyclic AMP standard
6. dibutyl thymidine triphosphate standard
7. dibutyl uridine triphosphate
8. Same as 4. Total SRFs released by 10^9 colony-forming-units of *L. monocytogenes* during 3½ hr in pH neutral buffer after an initial 12 hr exposure in pH neutral buffer.
9. Sephadex Peak I of SRFs <10kDa released by 10^9 colony-forming-units of *L. monocytogenes* for 12 hr in pH neutral buffer.
10. Same as 4.
11. dibutyl cyclic AMP
12. Total SRFs released by 10^9 colony-forming-units of *L. casei* during 8 hr in pH neutral buffer.
13. Total SRFs released by 10^9 colony-forming-units of *L. casei* during 24
14. Vacant.
15. dibutyl cyclic AMP

RESULTS & CONCLUSIONS:

Both *L. monocytogenes* and *L. casei* release ribonucleotides during exposures to pH neutral buffer. Their oligoribonucleotides <10kDa are similar.

3

[illegible]

8th yr

025

2 letter
4/27/74

15

 $\hookrightarrow \mathcal{P}_2$

38

0.04

0.13

0.43

0.54

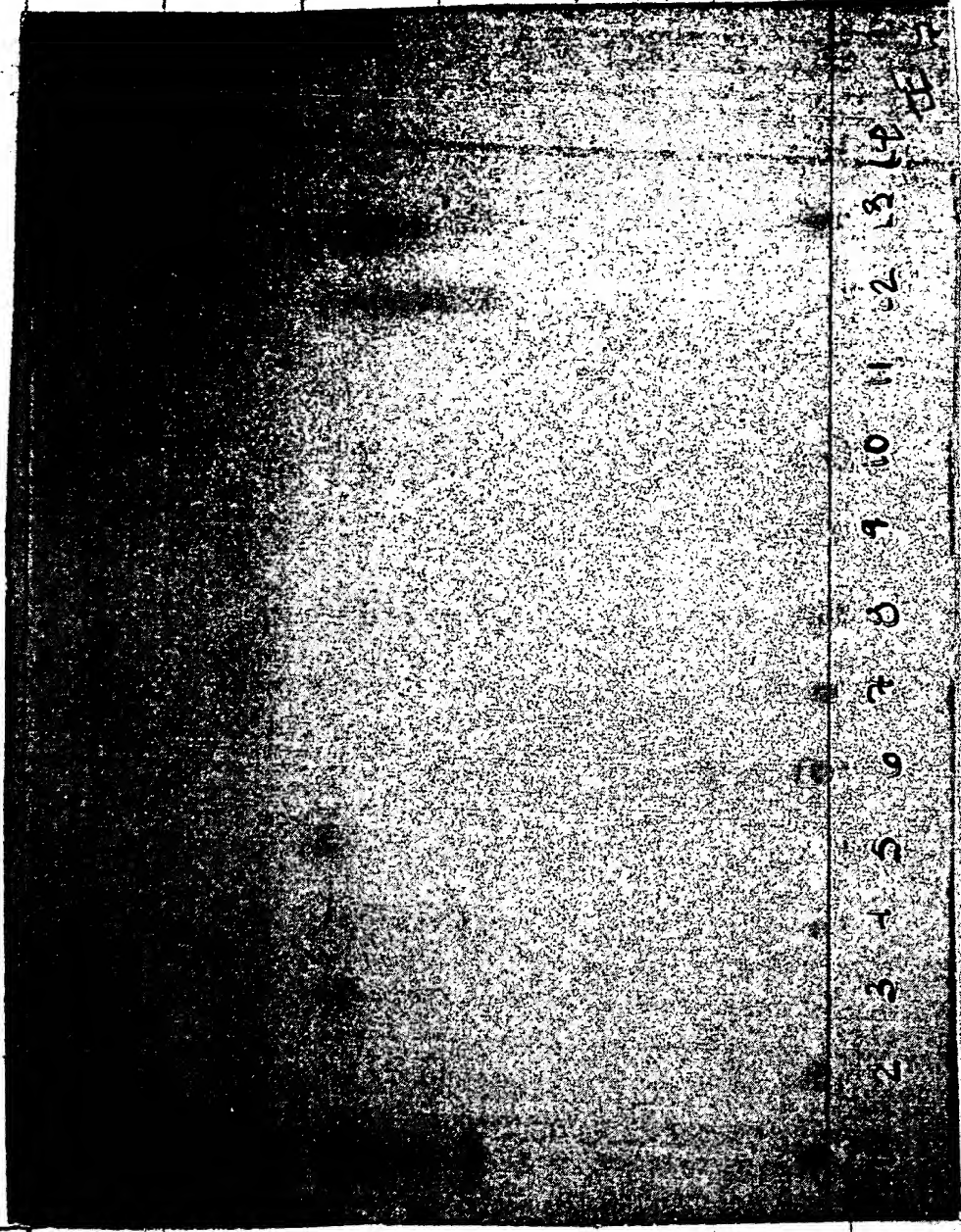
0.59 *

0.59
date
Index UV 11-
2 p. 30 for
top dots on TLC plate

Similar to breakdown product of dTTP (maybe dTMP_{prec} dTDP?)

8830 — * — 393

↓ ↓

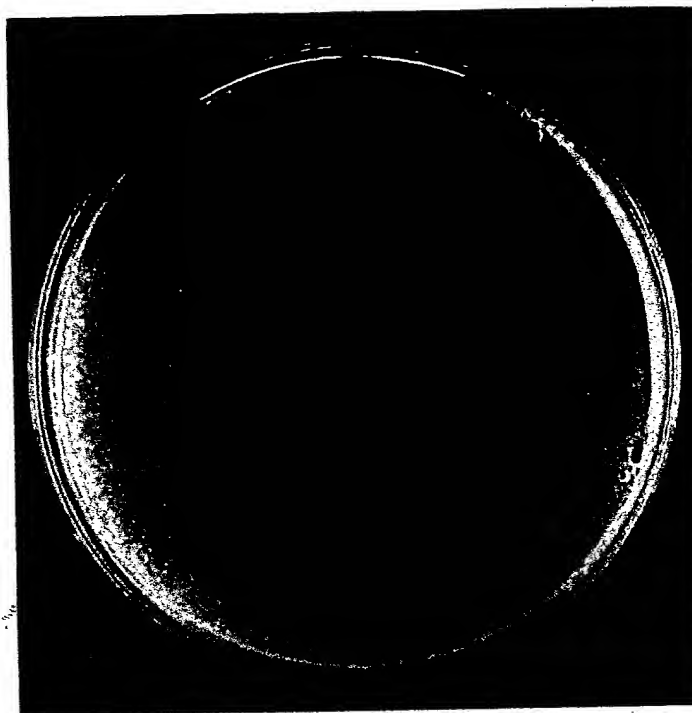


2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIGURE 1

SRFs Do Not Possess Bacteriocin-like Activity

L. monocytogenes, *L. plantarum* and *E. faecium* do not release bacteriocins by passage into pH neutral buffer or pH 4 buffer.



PROCEDURE:

A lawn of *Lactobacillus helveticus* ATCC 15009 was prepared by pour plating the test strain used by De Vuyst for the detection of bacteriocin.

Three hr later:

- 10 μ l containing 25 μ g of Nisin, a known bacteriocin from Sigma-Aldrich was added to the solidified agar at the top spot in the photograph, i.e., at 12 o'clock.
- 10 μ l of total SRFs released during 12 hr in pH neutral buffer by 10^9 colony-forming-units of *L. monocytogenes* per ml. were applied at site "1."
- 10 μ l of SRFs <10 kDa released during 12 hr in pH neutral buffer by 10^9 colony-forming-units of *L. monocytogenes* per ml. were applied at site "2."
- 10 μ l of SRFs <10 kDa released during 12 hr in pH 4 buffer by 10^9 colony-forming-units of a mixture of five strains of *L. plantarum* and two strains of *E. faecium* per ml. were applied at site "3."

RESULTS & CONCLUSIONS

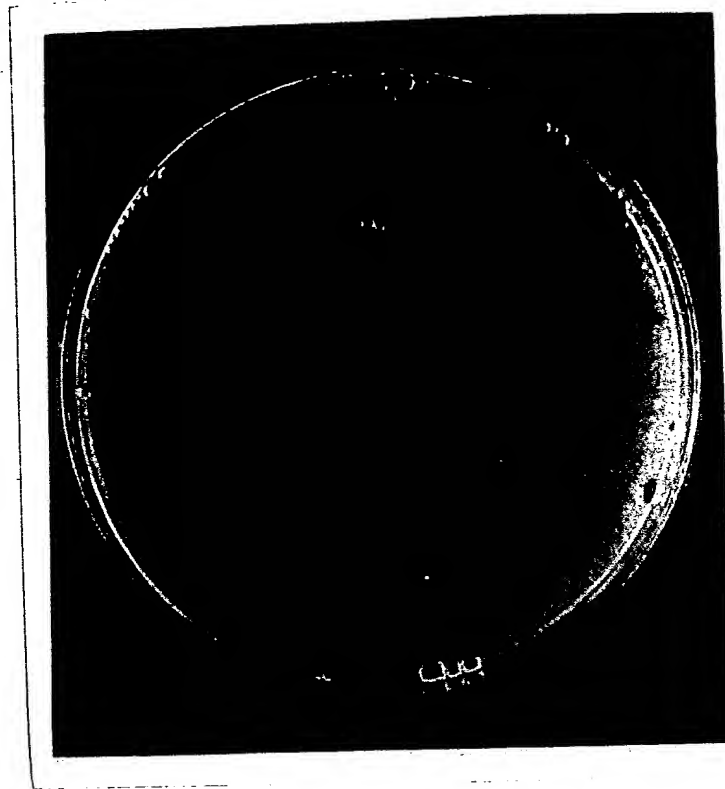
The clear area around the application point of Nisin demonstrates the inhibitory powers of bacteriocins.

No clear zones formed around the spots where SRFs were added indicate that SRFs do not possess bacteriocin or bacteriocin-like activity.

FIGURE 2

SRFs Do Not Possess Bacteriocin-like Activity

L. monocytogenes, *L. plantarum* and *E. faecium* do not release bacteriocins by exposure to pH neutral buffer or pH 4 buffer.



PROCEDURE:

A lawn of *Lactobacillus helveticus* ATCC 15009 was prepared by pour plating the test strain used by De Vuyst for the detection of bacteriocin.

Three hr later:

- 10 μ l containing 25 μ g of Nisin, a known bacteriocin, from Sigma-Aldrich was added to the solidified agar at the top spot in the photograph, i.e., at 12 o'clock.
- 10 μ l of SRFs <10 kDa released during 12 hr in pH neutral buffer by 10^{10} colony-forming-units of *L. monocytogenes* per ml. were applied at site "4."
- 10 μ l of SRFs <10 kDa released by 10^9 colony-forming-units of a mixture of five strains of *L. plantarum* and two strains of *E. faecium* per ml. first exposed to pH 4 for 3 hr, then pH 7 for 12 hr were applied at site "5."
- 10 μ l of SRFs <10 kDa released by 10^9 colony-forming-units of a heat killed mixture of five strains of *L. plantarum* and two strains of *E. faecium* per ml. first exposed to pH 4 for 3 hr, then pH 7 for 12 hr were applied at site "6."

RESULTS & CONCLUSIONS

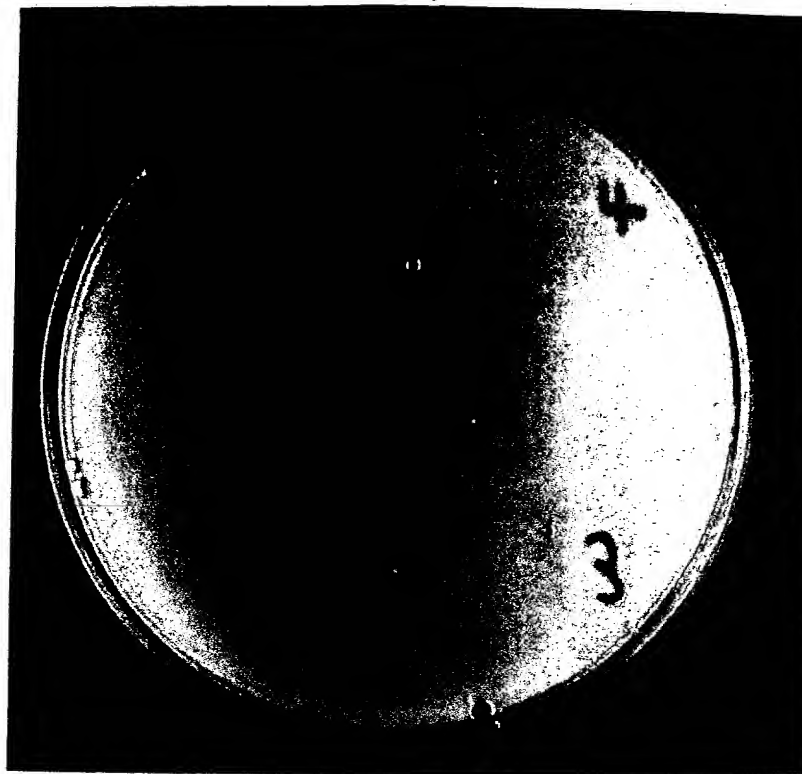
The clear area around the application point of Nisin demonstrates the ability of the bacteriocin to inhibit the growth of bacteria.

No clear zones formed around the spots where SRFs were added indicate that SRFs do not possess bacteriocin or bacteriocin-like activity.

FIGURE 3

SRFs Do Not Possess Bacteriocin-like Activity

L. casei, *L. plantarum* or *E. faecium* do not release bacteriocins during exposure to pH neutral buffer or pH 4 buffer.



PROCEDURE:

A lawn of *Lactobacillus helveticus* ATCC 15009 was prepared by pour plating the test strain used by De Vuyst for the detection of bacteriocin.

Three hr later:

- 10 μ l containing 25 μ g of Nisin, a known bacteriocin from Sigma-Aldrich was added to the solidified agar at the top spot in the photograph, i.e., at 12 o'clock.
- 10 μ l of total SRFs released by 10^9 colony-forming-units of *L. casei* per ml during 12 hr in pH neutral buffer were applied at site "1."
- 10 μ l of SRFs <10 kDa released by 10^9 colony-forming-units of *L. casei* per ml during 12 hr in pH neutral buffer were applied at site "2."
- 10 μ l of total SRFs released by 10^9 colony-forming-units of a mixture of 5 strains of *L. plantarum* per ml and 2 strains of *E. faecium* during 12 hr in pH 7 buffer were applied at site "3."
- 10 μ l of SRFs <10 kDa released by exposing 10^9 colony-forming-units of a mixture of 5 strains of *L. plantarum* per ml and 2 strains of *E. faecium* during 12 hr in pH 7 buffer were applied at site "4."

RESULTS & CONCLUSIONS

The clear area around the application point of Nisin demonstrates the ability of bacteriocin to inhibit the growth of bacteria.

No clear zones formed around the spots where SRFs were added indicate that SRFs do not possess bacteriocin or bacteriocin-like activity.